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TITLE OF THE INVENTION (280 characters max)

COMPOUNDS HAVING INHIBITORY ACTIVITY TOWARDS PHOSPHATIDLYLINOSITOL 3-KINASE AND METHODS OF USE THEREOF

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Respectfully submitted,

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X Additional inventors are being named on separately numbered sheets attached hereto

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Respectfully submitted this 13th day of June, 2003.

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UNITED STATES PROVISIONAL PATENT APPLICATION

of

DREES et al.

for

COMPOUNDS HAVING INHIBITORY ACTIVITY TOWARDS PHOSPHATIDYLINOSITOL 3-KINASE AND METHODS OF USE THEREOF

TO THE COMMISSIONER OF PATENTS AND TRADEMARKS:

Your petitioners, Beth E. Drees, citizen of the United States, whose residence and postal mailing address is 603 Sixth Avenue, Salt Lake City, Utah 84103, and Leena Chakravarty, citizen of India, whose residence and postal mailing address is 9462 S. Hunts End Drive, Sandy, Utah 84092, and Glenn D. Prestwich, citizen of the United States, whose residence and postal mailing address is 1500 South Sunnydale Lane, Salt Lake City, Utah 84108 and Mr. Gyorgy Dorman, citizen of Hungary, whose residence and postal mailing address is Kondorsi ut 80, Budapest, H-1119, Hungary and Mariann Kavecz, citizen of Hungary, whose residence and postal mailing address is Erdody-Palffy Tamas u.46, Varpalota, H-8100, Hungary and Andras Lukacs whose residence and postal mailing address is Wesselenyl u.69. 1/3, H-1077, Budapest, Hungary, and Laszlo Urge, a citizen of Hungary, whose residence and postal mailing address is Kerekgyarto u.3, H-1147, Budapest, Hungary, and Ferenc Darvas, citizen of Hungary, whose residence and postal mailing address is Zapor u.11/B, Budapest, H-1034, Hungary, prays that they may preserve their rights to letters patent by this provisional patent application as the inventors of a **COMPOUNDS HAVING INHIBITORY ACTIVITY TOWARDS PHOSPHATIDYLINOSITOL 3-KINASE AND METHODS OF USE THEREOF** as set forth in the following specification.

COMPOUNDS HAVING INHIBITIVE ACTIVITY OF PHOSPHATIDYLINOSITOL 3-KINASE AND METHODS OF USE THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to phosphatidylinositol 3-kinase (PI 3-K) enzymes, and more particularly to inhibitors of PI 3-K activity and to methods of using such materials.

Related Art

The behavior of all cellular communications is governed by signaling systems which translate external signals such as hormones, neurotransmitters, and growth factors into intracellular second messengers. Phosphoinositide polyphosphates (PIPn) are key lipid second messengers in cellular signaling (Martin, Ann. Rev. Cell Dev. Biol., 14:231-2614 (1998)). Because their activity is determined by their phosphorylation state, the enzymes that modify these lipids are central to the correct execution of signaling events (Leslie, et al., Chem Rev, 101:2365-80. (2001)). Disruptions in these processes are common to many disease states, including cancer, diabetes, inflammation, and cardiovascular disease.

The production of the phosphoinositide polyphosphate $PI(3,4,5)P_3$ or PIP_3 by phosphatidylinositol 3-kinase (PI 3-K) is important in pathways governing cell proliferation, differentiation, apoptosis, and migration (MacDougall, et al., Curr Biol, 5:1404-15. (1995, Carpenter and Cantley, Biochim Biophys Acta, 1288:M11-6. (1996, Derman, et al., J Biol Chem, 272:6465-70. (1997, Toker and Cantley, Nature, 387:673-6. (1997, Rameh and Cantley, J Biol Chem, 274:8347-50. (1999)). Alterations which affect correct regulation of PIP_3 levels and the levels of their lipid products are associated with a variety of cancer types (Phillips et al., Cancer 83:41-47. (1998) ,Shayesteh, et al., Nat Genet, 21:99-102. (1999), Ma, et al., Oncogene, 19:2739-44. (2000)). Mutations which affect the regulation of PI 3-K signaling contribute to abnormal proliferation and tumorigenesis (Li, et al., Science, 275:1943-7. (1997), Teng, et al., Cancer Res, 57:5221-5. (1997)) (Shayesteh, et al., Nat Genet, 21:99-102. (1999), Ma, et al., Oncogene, 19:2739-44. (2000)). .

When activated by tyrosine kinase receptors in response to growth factor stimulation, PI 3-K catalyzes the formation of PIP_3. By increasing cellular levels of PIP_3, PI 3-K induces the formation of defined molecular complexes that act in signal transduction pathways. Most

notably, PI 3-K activity suppresses apoptosis and promotes cell survival through activation of its downstream target, PKB/Akt (Franke, et al., Cell, 81:727-36. (1995), Datta, et al., J Biol Chem, 271:30835-9. (1996)). The lipid phosphatases PTEN (Maehama and Dixon, Trends Cell Biol, 9:125-8. (1999)) and SHIP (Liu, et al., Genes Dev, 13:786-91. (1999)) are two enzymes that both act to decrease the cellular levels of PIP_3 by conversion either to $PI(4,5)P_2$ or $PI(3,4)P_2$.

Presently, the PI 3-kinase enzyme family has been divided into three classes based on their substrate specificities. Class I PI 3-Ks can phosphorylate phosphatidylinositol (PI), phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-biphosphate (PIP2) to produce phosphatidylinositol-3-phosphate (PIP), phosphatidylinositol-3,4-biphosphate, and phosphatidylinositol-3,4,5-triphosphate, respectively. Class II PI 3-Ks phosphorylate PI and phosphatidylinositol-4-phosphate, whereas Class III PI 3-Ks can only phosphorylate PI. Eight separate isoforms of PI 3-K have been characterized in humans.

The initial purification and molecular cloning of PI 3-kinase revealed that it was a heterodimer consisting of p85 and p110 subunits (Otsu et al., Cell, 65:91-104 (1991); Hiles et al., Cell, 70:419-29 (1992)). Since then, four distinct Class I PI 3-Ks have been identified, designated PI 3-K alpha., beta., delta., and gamma., each consisting of a distinct 110 kDa catalytic subunit and a regulatory subunit. More specifically, three of the catalytic subunits, i.e., p110 alpha., p110 beta. and p110 delta., each interact with the same regulatory subunit, p85; whereas p110 gamma. interacts with a distinct regulatory subunit, p101. In each of the PI3-Kinase alpha, beta, and delta subtypes, the p85 subunit acts to localize PI 3-kinase to the plasma membrane by the interaction of its SH2 domain with phosphorylated tyrosine residues (present in an appropriate sequence context) in target proteins (Rameh et al., Cell, 83:821-30 (1995)). Two isoforms of p85 have been identified, p85.alpha., which is ubiquitously expressed, and p85.beta., which is primarily found in the brain and lymphoid tissues (Volinia et al., Oncogene, 7:789-93 (1992)). Association of the p85 subunit to the PI 3-kinase p110 alpha., beta., or delta. catalytic subunits appears to be required for the catalytic activity and stability of these enzymes. In addition, the binding of Ras proteins also upregulates PI 3-kinase activity. Though a wealth of information has been accumulated in recent past on the cellular functions of PI 3-kinases in general, in particular for PI 3-K alpha and PI 3-K gamma, the roles played by the individual isoforms are have yet to be clearly defined.

Phosphatidylinositol 3-kinase alpha is composed of 85-kD (171833) and 110-kD subunits. The 85-kD subunit lacks PI 3-kinase activity and acts as an adaptor, coupling the 110-kD subunit (p110) to activated protein tyrosine kinases. In COS-1 cells, p110alpha was

catalytically active only when complexed with p85alpha (Hiles, et al., Cell 70: 419-429, (1992)) Volinia et al. (Genomics, 24: 472-477 (1994)) cloned the cDNA for the human p110 subunit (PIK3CA). The 5.3-kb p110-alpha transcript is detectable by Northern blot in human pancreas, skeletal muscle, liver, and heart.

PI 3-kinase gamma was identified by Stoyanov et al. (1995) from a screen of a human bone marrow cDNA library with primers based on the sequences of yeast and bovine PIK3 p110 subunits. They isolated a human cDNA for a novel p110 subunit, which they termed p110-gamma. The cDNA encodes a predicted 120-kD, 1,050-amino acid polypeptide with 36% identity to human p110-alpha. Recombinant p110-gamma did not interact with the p85 subunit in vivo, in contrast to recombinant p110-alpha (Stoyanov, et al., Science, 269:690-3. (1995)). The p110 gamma enzyme was activated in vitro by both the alpha and beta gamma subunits of heterotrimeric guanosine triphosphate (GTP)-binding proteins (G proteins) rather than by interaction with p85. In contrast, the p85-dependent p110-alpha is not similarly affected by the G protein subunits. The p110-gamma isotype may link signaling through G protein-coupled receptors and generate phosphoinositide second messengers phosphorylated in the D-3 position (Stoyanov, et al., Science, 269:690-3. (1995)).

The catalytic subunit of PI 3-K beta, p110 beta, was identified via RT-PCR with RNA from a T-lymphocyte cell line and degenerate primers based on regions conserved between a bovine and a yeast PI 3-K. A partial cDNA that encoded a human PI 3-K was recovered and used to isolate additional cDNAs from an embryonic kidney cell line (293) library (Hu et al., Molec. Cell. Biol., 13:7677-7688, (1993). The sequence of the predicted 1,070-amino acid protein, p110-beta, was 42% identical to that of bovine p110. Epitope tagged p110-beta has PI 3-K activity, and antibodies against p110-beta immunoprecipitated an endogenous PI3K activity from cell lysates. Both the epitope-tagged protein and endogenous p110-beta associated with the 85-kD subunit in vivo. Northern blot analysis revealed that the major 4.8-kb p110-beta transcript is expressed in several human and rodent cell lines, as well as in all mouse tissues tested (Hu et al., Molec. Cell. Biol., 13:7677-7688, (1993).

Identification of the p110 delta. isoform of PI 3-kinase is described in Chantry et al., J Biol Chem, 272:19236-41 (1997). It was observed that the human p110.delta. isoform is expressed in a tissue-restricted fashion. It is expressed at high levels in lymphocytes and lymphoid tissues, suggesting that the protein might play a role in PI 3-kinase-mediated signaling in the immune system. Mice expressing a catalytically inactive form of PI 3-K delta exhibited impaired signaling and attenuated immune responses by B and T cell antigen receptors (Okkenhaug et al., Science, 297:1031-1034, (2002)). The mutant mice also developed

inflammatory bowel disease. Since the IBD7 (inflammatory bowel disease) susceptibility locus maps to chromosome 1p36, in the region containing PIK3CD, the gene encoding p110 delta, it has been suggested that PIK3CD may be a candidate susceptibility gene. Details concerning the p110 isoform also can be found in U.S. Pat. Nos. 5,858,753; 5,822,910; and 5,985,589.

Specific inhibitors against individual members of a family of enzymes provide invaluable tools for deciphering the functions of each enzyme. Experimental usage of PI 3-K inhibitors has contributed to the current understanding of the role of PI 3-K activity in normal function and in disease. The major pharmacological tools used in this capacity are wortmannin (Powis, et al., Cancer Res, 54:2419-23. (1994)), and bioflavenoid compounds, including quercetin (Matter et al., Biochem. Biophys. Res. Commun. 186:624-631. (1992)) and LY294002 (Vlahos, et al., J Biol Chem, 269:5241-8. (1994)). The concentrations of wortmannin needed to inhibit PI 3-Ks range from 1-100 nM, and inhibition occurs via covalent modification of the catalytic site (Wymann et al., Mol. Cell. Biol. 16:1722-1733. (1996)). The bioflavenoid quercetin effectively inhibits PI 3-K with an IC_{50} of 3.8 μM, but has poor selectivity, as it shows inhibitory activity toward PI 4-kinase, and several protein kinases (Matter et al., Biochem. Biophys. Res. Commun. 186:624-631. (1992)). LY294002 is a synthetic compound made using quercetin as a model, inhibits PI 3-K with an IC_{50} of 100 μM (Vlahos, et al., J Biol Chem, 269:5241-8. (1994)). Both quercetin and LY294002 are competitive inhibitors of the ATP binding site of PI 3-K, however, only LY294002 shows specificity for inhibition of PI 3-K and does not affect other types of kinases. Both wortmannin and LY294002 have been used extensively to characterize the biological roles of PI 3-K, however, neither shows selectivity for individual PI 3-K isoforms. Hence, the utility of these compounds in studying the roles of individual Class I PI 3-kinases is limited.

The PI 3-K inhibitors are expected to be a new type of medicament useful for cell proliferation disorders, in particular as antitumor agents. As PI 3-K inhibitors, wortmannin [H. Yano et al., J. Biol. Chem., 263, 16178 (1993)] and LY294002 [J. Vlahos et al., J. Biol. Chem., 269, 5241(1994)] which is represented by the formula below, are known. However, creation of PI 3-K inhibitors having more potent cancer cell growth inhibiting activity is desired.

There is evidence that PI 3-K inhibitors may have therapeutic potential in inflammation and autoimmune disease. In particular, inhibitors of the p110 delta and p110 gamma isoforms, which show largely restricted expression to leukocytes and appear to act in immune regulation, may be of therapeutic value. Based on studies using wortmannin, there is evidence that PI 3-kinase function is also required for some aspects of leukocyte signaling through G-protein

coupled receptors (Thelen et al., Proc Natl Acad Sci USA, 91:4960-64 (1994)). Moreover, it has been shown that wortmannin and LY294002 block neutrophil migration and superoxide release.

PI 3-kinase appears to be involved in a number of aspects of leukocyte activation. A p85-associated PI 3-kinase activity has been shown to physically associate with the cytoplasmic domain of CD28, which is an important costimulatory molecule for the activation of T-cells in response to an antigen (Pages et al., Nature, 369:327-29 (1994); Rudd, Immunity, 4:527-34 (1996)). Activation of T cells through CD28 lowers the threshold for activation by an antigen and increases the magnitude and duration of the proliferative response. These effects are linked to increases in the transcription of a number of genes including interleukin-2 (IL2), an important T cell growth factor (Fraser et al., Science, 251:313-16 (1991)). Mutation of CD28 such that it can no longer interact with PI 3-kinase leads to a failure to initiate IL2 production, suggesting a critical role for PI 3-kinase in T cell activation.

PI 3-K activity is polarized at the leading edge of migrating leukocytes (Wang et al., Nat. Cell Biol. 4:513-518 (2002), Weiner et al., Nat. Cell Biol. 4:509-512. (2002)), and PI 3-K activation is a critical early event in leukocyte migration in response to chemokines and other chemoattractants, which is a hallmark of the inflammatory response. Studies using PI 3-K gamma knockout mice show that p110 gamma is required for PIP_3 production, PKB activation, and superoxide production in neutrophils following activation of G-coupled protein receptors by chemoattractants (Sasaki et al., Science 287:1040-1044. (2000), Hirsch et al., Science 287:1049-1053. (2000)). Chemotaxis of neutrophils, macrophages, and T lymphocytes is impaired in the absence of PI 3-K gamma (Li et al., Science 287:1046-1049. (2000)). Mice expressing a catalytically inactive form of PI 3-K delta exhibited impaired signaling and attenuated immune responses by B and T cell antigen receptors (Okkenhaug et al., Science, 297:1031-1034, (2002)). In addition, mice lacking the 5' phosphoinositide SHIP, which converts PIP_3 to $PI(3,4)P_2$, exhibit lethal infiltration of the lungs by macrophages and neutrophils (Helgason et al., Genes Dev. 12:1610-1620. (1998)), indicating that constitutively high levels of PIP_3 might produce excessive inflammation. Thus, selective inhibitors of PI 3-K gamma and/or delta could potentially be useful in blocking inflammatory response in asthma, rheumatoid arthritis, multiple sclerosis, and other immune disorders.

In addition, PI 3-K inhibitors may be useful in treating some aspects of cardiovascular disease. Cardiomyocyte-specific inactivation of PTEN in mice resulted in hypertrophy and a dramatic decrease in cardiac contractility (Crackower, et al., Cell, 110:737-49. (2002)). Analysis of PTEN/PI-3K-gamma double-mutant mice revealed that the cardiac hypertrophy and

contractility defects could be genetically uncoupled. PI-3K-alpha was found to mediate the alteration in cell size, while PI-3K-gamma was found to act as a negative regulator of cardiac contractility. PI 3-K gamma was shown to be expressed in cardiomyocytes, and to regulate cardiac function by negative regulation of cAMP levels in response to G-coupled β2 adrenergic receptor stimulation, with downregulation of cAMP levels resulting in decreased contractility. Thus, selective inhibition of PI 3-K gamma may allow induction of improved cardiac contractility during heart failure. PI 3-Ks also appear to act in regulating vascular tone in hypertension. Aortas of hypertensive rats show increased PI 3K activity and elevated expression of p110 beta and p110 delta isoforms, and treatment of hypertensive rats with the PI 3-K inhibitors LY294002 and wortmannin attenuated symptoms of hypertension (Sata and Nagai, Circ Res. 91:273-275. (2002)). Selective inhibitors of these PI 3-K isoforms may be useful for treatment of hypertension.

Because many oncogenic signaling pathways are mediated by PI 3-K, inhibitors that target PI 3-K activity may have application for the treatment of cancer. Studies using comparative genomic hybridization revealed several regions of recurrent abnormal DNA sequence copy number (reviewed by Knuutila et al., Am. J. Path. 152: 1107-1123, (1998)) that may encode genes involved in the genesis or progression of ovarian cancer. One region found to be increased in copy number in approximately 40% of ovarian and other cancers contains the PIK3CA gene, which encodes the p110 alpha catalytic subunit of PI 3-K alpha. This association between PIK3CA copy number and PI3-kinase activity made PIK3CA a candidate oncogene because a broad range of cancer-related functions had been associated with PI 3-kinase-mediated signaling. PIK3CA is frequently increased in copy number in ovarian cancers, and increased copy number is associated with increased PIK3CA transcription, p110-alpha protein expression, and PI 3-kinase activity (Shayesteh, et al., Nature Genet. 21: 99-102, (1999)). Furthermore, treatment of ovarian cancer cell lines exhibiting increased PI 3-K activity and Akt activation with a PI 3-kinase inhibitor decreased proliferation and increased apoptosis (Shayesteh, et al., Nature Genet. 21: 99-102, (1999), Yuan et al., Oncogene 19:2324-2330. (2000)). Thus, PI 3-K alpha has an important role in ovarian cancer. In cervical cancer cell lines harboring amplified PIK3CA, the expression of the gene product was increased and was associated with high PI 3-kinase activity (Ma et al.,Oncogene 19: 2739-2744, (2000)). Thus, increased expression of PI 3-kinase alpha in cervical cancer may promote cell proliferation and reduce apoptosis. In addition, mutation of the lipid phosphatase and tumor suppressor PTEN, a 3' phosphatase that breaks down PIP_3, is one of the most common cancer-associated mutations, and is particularly associated with glioblastoma, prostate, endometrial, and breast cancers (Li et al., Science

275:1943-1947 (1997), Teng et al., Cancer Res. 57:5221-5225. (1997), Ali et al., J. National Cancer Institute, 91:1922-1932. (1999), Simpson and Parsons, Exp. Cell Res. 264:29-41 (2002)). PI 3-K activity suppresses apoptosis and promotes cell survival largely through activation of its downstream target, PKB/Akt (Franke et al. Cell 81:727-736. (1995), Dattaet al., J Biol Chem 271:30835-30839 (1996)). Akt activation and amplification is present in many cancers (Testa and Bellicosa, Proc. Natl. Acad. Sci. USA 98:10983-10985. (2002)).

Treatment with PI 3-K inhibitors has been shown to block proliferation of several cancer cell lines, and to be effective in tumor xenograft models in addition to ovarian carcinoma. Akt is activated in a majority of non-small cell lung cancer cell lines, and treatment with PI 3-K inhibitors causes proliferative arrest in these cells (Brognard et al., Cancer Res. 60:6353-6358. (2000), Lee et al., J. Biol. Chem. electronic publication, (2003)). The PI 3-K/Akt pathway is also constitutively activated in a majority of human pancreatic cancer cell lines, and treatment with PI 3-K inhibitors induced apoptosis in these cell lines. Decreased tumor growth and metastasis was also observed upon treatment with PI 3-K inhibitors in a xenografts model of pancreatic cancer (Perugini et al., J. Surg. Res. 90:39-44 (2000), Bondar et al., Mol. Cancer Ther. 1:989-997 (2002)). Treatment with LY204002 induced growth arrest and apoptosis in PTEN-deficient human malignant glioma cells (Shingu et al., J. Neurosurg. 98:154-161. (2003)). LY294002 produces growth arrest in human colon cancer cell lines and suppression of tumor growth in colon carcinoma xenografts in mice (Semba et al., Clin Cancer Res. 8:1957-1963. (2002)). Inhibitors of PI 3-K inhibit *in vitro* anchorage-independent growth and *in vivo* metastasis of liver cancer cells (Nakanishi et al., Cancer Res. 62:2971-2975. (2002)). Treatment of Burkitt's lymphoma cells with LY294002 induces apoptosis (Brennan et al., Oncogene 21:1263-1271. (2002)). LY294002 also has been shown to induce apoptosis in multi-drug resistant cells (Nicholson et al., Cancer Lett. 190:31-36. (2003)). Thus, PI 3-K inhibitors may be suitable therapeutics for many tumors exhibiting activated or increased levels of PI 3-K or PKB/Akt as well as tumors which are PTEN-deficient.

Several studies have demonstrated that agents which target the PI 3-K pathway can enhance the effects of standard chemotherapeutic agents in a variety of cancer types. Thus, PI 3-K inhibitors may have value as novel adjuvant therapies for certain cancers. PI 3-K inhibitors induce apoptosis in pancreatic carcinoma cells exhibiting constitutive phosphorylation and activation of AKT, and suboptimal doses produce additive inhibition of tumor growth when combined with a suboptimal dose of gemcitabine (Ng, et al., Cancer Res, 60:5451-5. (2000, Bondar, et al., Mol Cancer Ther, 1:989-97. (2002)). Inhibition of PI 3-K also increases the responsiveness of pancreatic carcinoma cells to the non-steroidal anti-inflammatory agent

(NSAID) sulindac (Yip-Schneider, et al., J Gastrointest Surg, 7:354-63. (2003)). In a mouse xenograft model of pancreatic cancer, a combination of wortmannin with gemcitabine also showed increased efficacy in induction of tumor apoptosis relative to treatment with each agent alone (Ng, et al., Clin Cancer Res, 7:3269-75. (2001)). In an athymic mouse xenograft model of ovarian cancer, combined treatment with LY294002 and paclitaxal results in increased efficacy of paclitaxal-induced apoptosis of tumor cells, and allows the use of decreased levels of LY294002, resulting in less dermatological toxicity (Hu, et al., Cancer Res, 62:1087-92. (2002)). HL60 human leukemia cells show sensitization to cytotoxic drug treatment and Fas-induced apoptosis when treated with PI 3-K inhibitors, suggesting a role for PI 3-K inhibition in treating drug resistant acute myeloid leukemia (O'Gorman, et al., Leukemia, 14:602-11. (2000, O'Gorman, et al., Leuk Res, 25:801-11. (2001)). Inhibition of PI 3-K enhances the apoptotic effects of sodium butyrate, gemcitabine, and 5-fluoruracil in an aggressive colon cancer cell line (Wang, et al., Clin Cancer Res, 8:1940-7. (2002)). LY294002 potentiates apoptosis induced by doxorubicin, trastumazab, paclitaxal, tamoxifen, and etoposide in breast cancer cell lines exhibiting PTEN mutations or erbB2 overexpression (Clark, et al., Mol Cancer Ther, 1:707-17. (2002)). Inhibition of PI 3-K potentiates the effect of etoposide to induce apoptosis in small cell lung cancer cells (Krystal, et al., Mol Cancer Ther, 1:913-22. (2002)).

In addition to enhancing the effects of chemotherapeutic agents for cancer treatment, PI 3-K inhibitors also may enhance tumor response to radiation treatment. Inhibitors of PI 3-K revert radioresistance in breast cancer cells transfected with constitutively active H-ras (Liang, et al., Mol Cancer Ther, 2:353-60. (2003)), and PI 3-K inhibitors enhance radiation-induced apoptosis and cytotoxicity in tumor vascular endothetial cells (Edwards, et al., Cancer Res, 62:4671-7. (2002)). Thus, PI 3-K inhibitors could be used to enhance response to radiotherapy, both in tumor cells and in tumor vasculature.

US Patent No. 6,403,588 discloses imidazopyridine derivatives having excellent PI 3-K inhibiting activity and cancer cell growth inhibiting activity. US Patent No. 5,518,277 discloses compounds that inhibit PI 3-Kdelta activity, including compounds that selectively inhibit PI 3-Kdelta activity. However, all of these compounds have a structure different from those of the present invention.

SUMMARY OF THE INVENTION

It has been recognized that it would be advantageous to develop inhibitors of PI 3-K isozymes, in order that the functions of each isozyme can be better characterized. In particular,

inhibitors of PI 3-K are desirable for exploring the roles of PI 3-K isozymes and for development of pharmaceuticals to modulate the activity of the isozymes.

One aspect of the present invention is to provide compounds that can inhibit the biological activity of human PI 3-Kalpha. Another aspect of the invention is to provide compounds that inhibit other PI 3-K isoforms, including PI 3-K beta, gamma and delta. Another aspect of the invention is to provide methods of synthesizing and using these PI 3-K inhibitors.

The present invention further relates to novel pharmaceutical compositions, particularly to PI 3-K inhibitors and antitumor agents, comprising a compound of the present invention and a pharmaceutically acceptable carrier.

A further aspect of the present invention relates to treatment methods of disorders (especially cancers) influenced by PI 3-K, wherein an effective amount of a compound of the present invention is administered to humans or animals.

Additional features and advantages of the invention will be apparent from the detailed description which follows, taken in conjunction with the accompanying drawings, which together illustrate, by way of example, features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

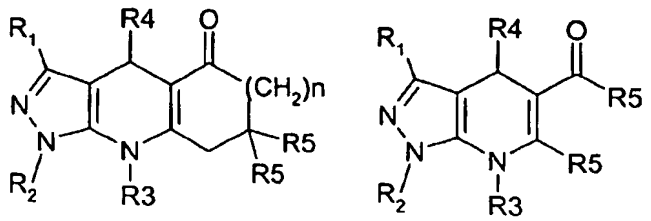
FIG. 1 illustrates the effect of the compounds of the present invention on paired tumor cell lines;

FIG. 2 illustrates the selective anti-proliferative effects of the PI-3-K inhibitors of the present invention.

DETAILED DESCRIPTION

Reference will now be made to the exemplary embodiments illustrated in the drawings, and specific language will be used herein to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended. Alterations and further modifications of the inventive features illustrated herein, and additional applications of the principles of the inventions as illustrated herein, which would occur to one skilled in the relevant art and having possession of this disclosure, are to be considered within the scope of the invention.

The present invention relates to novel compounds or scaffolds which are useful as PI 3-K inhibitors and antitumor agents. The compounds of the present invention are represented by one of the following general formula:



wherein R_1 and R_2 are each independently a member selected from the group consisting of alkyl, aryl, hetaryl substituted alkyl, aryl, aralkyl and hetaryl groups;

R_3 can be $CO\text{-}R_5$, or $SO_2\text{-}R_5$; $CO\text{-}O\text{-}R_5$, $CO\text{-}N\text{-}R_4$, R_5, and a member selected from the group consisting of H, alkyl, aralkyl substituted alkyl and aralkyl groups;

R_4 and R_5 can be a member selected from the group consisting of H, alkyl, substituted alkyl, cycloalkyl and aralkyl groups.

The terms "substituted alkyl, cycloalkyl, alkenyl, or aralkyl" means: $C_{1\text{-}15}$ alkyl, $C_{3\text{-}8}$ cycloalkyl, $C_{2\text{-}18}$ alkenyl or aralkyl groups which may be substituted by 1 to 5 substituents selected from the group consisting of (i) nitro, (ii) hydroxy, (iii) cyano, (iv) carbamoyl, (v) mono- or di-$C_{1\text{-}4}$ alkyl-carbamoyl, (vi) carboxy, (vii) $C_{1\text{-}4}$ alkoxy-carbonyl, (viii) sulfo, (ix) halogen, (x) $C_{1\text{-}4}$ alkoxy, (xi) phenoxy, (xii) halophenoxy, (xiii) $C_{1\text{-}4}$ alkylthio, (xiv) mercapto, (xv) phenylthio, (xvi) pyridylthio, (xvii) $C_{1\text{-}4}$ alkylsulfinyl, (xviii) $C_{1\text{-}4}$ alkylsulfonyl, (xix) amino, (xx) $C_{1\text{-}3}$ alkanoylamino, (xxi) mono- or di-$C_{1\text{-}4}$ alkylamino, (xxii) 4- to 6-membered cyclic amino, (xxiii) $C_{1\text{-}3}$ alkanoyl, (xxiv) benzoyl and (xxv) 5- to 10-membered heterocyclic group.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

The term "alkyl" means a straight or branched hydrocarbon chain having 1 to 15, preferably 1 to 6 carbon atoms, and is more preferably a methyl or ethyl group.

The term "aryl" is used throughout the specification to mean an aromatic cyclic hydrocarbon group. An aryl having 6 to 14 carbon atoms is preferable. It may be partially saturated. Preferred examples of such aryls are phenyl and naphthyl groups.

The term "hetaryl" is used throughout the specification to mean a 5- or 6-membered monocyclic heterocyclic group containing 1 to 4 hetero-atoms selected from oxygen, sulfur and nitrogen or a fused bicyclic heterocyclic group containing 1 to 6 hetero-atoms selected from oxygen, sulfur and nitrogen, each of which may be substituted by 1 to 4 substituents selected from the group consisting of (i) halogen, (ii) $C_{1\text{-}4}$ alkyl, (iii) $C_{1\text{-}4}$ haloalkyl, (iv) $C_{1\text{-}4}$ haloalkoxy,

(v) C_{1-4} alkoxy, (vi) C_{1-4} alkylthio, (vii) hydroxy, (viii) carboxy, (ix) cyano, (x) nitro, (xi) amino, (xii) mono- or di-C_{1-4} alkylamino, (xiii) formyl, (xiv) mercapto, (xv) C_{1-4} alkyl-carbonyl, (xvi) C_{1-4} alkoxy-carbonyl, (xvii) sulfo, (xviii) C_{1-4} alkylsulfonyl, (xix) carbamoyl, (xx) mono- or di-C_{1-4} alkyl-carbamoyl, (xxi) oxo and (xxii) thioxo.

The term "substituted aryl" is used throughout the specification to mean a C_{6-14} aryl group which may be substituted by 1 to 4 substituents selected from the group consisting of (i) halogen, (ii) C_{1-4} alkyl, (iii) C_{1-4} haloalkyl, (iv) C_{1-4} haloalkoxy, (v) C_{1-4} alkoxy, (vi) C_{1-4} alkylthio, (vii) hydroxy, (viii) carboxy, (ix) cyano, (x) nitro, (xi) amino, (xii) mono- or di-C_{1-4} alkylamino, (xiii) formyl, (xiv) mercapto, (xv) C_{1-4} alkyl-carbonyl, (xvi) C_{1-4} alkoxy-carbonyl, (xvii) sulfo, (xviii) C_{1-4} alkylsulfonyl, (xix) carbamoyl, (xx) mono- or di-C_{1-4} alkyl-carbamoyl, (xxi) oxo and (xxii) thioxo.

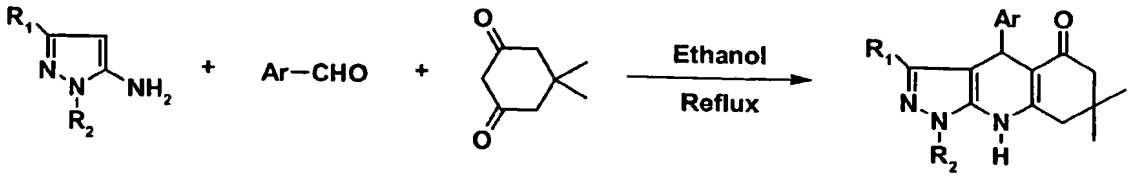
The term "substituted hetaryl" is used throughout the specification to mean hetaryl as described above may be substituted by 1 to 4 substituents selected from the group consisting of (i) halogen, (ii) C_{1-4} alkyl, (iii) C_{1-4} haloalkyl, (iv) C_{1-4} haloalkoxy, (v) C_{1-4} alkoxy, (vi) C_{1-4} alkylthio, (vii) hydroxy, (viii) carboxy, (ix) cyano, (x) nitro, (xi) amino, (xii) mono- or di-C_{1-4} alkylamino, (xiii) formyl, (xiv) mercapto, (xv) C_{1-4} alkyl-carbonyl, (xvi) C_{1-4} alkoxy-carbonyl, (xvii) sulfo, (xviii) C_{1-4} alkylsulfonyl, (xix) carbamoyl, (xx) mono- or di-C_{1-4} alkyl-carbamoyl, (xxi) oxo and (xxii) thioxo.

The compounds of the present invention may be geometric isomers or tautomers depending upon the type of substituents. The present invention also covers these isomers in separated forms and the mixtures thereof. Furthermore, some of the compounds may contain an asymmetric carbon in the molecule; in such case isomers could be present. The present invention also embraces mixtures of these optical isomers and the isolated forms of the isomers.

Some of the compounds of the invention may form salts. There is no particular limitation so long as the formed salts are pharmacologically acccptable. Specific examples of acid addition salts are salts of inorganic acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, nitric acid, phosphoric acid, etc., organic acids such as formic acid, acetic acid, propionic acid, oxalic acid, malonic acid, succinic acid, fumaric acid, maleic acid, lactic acid, malic acid, tartaric acid, citric acid, methanesulfonic acid, ethanesulfonic acid, aspartic acid, glutamic acid, etc. Specific examples of basic salts include salts with inorganic bases containing metals such as sodium, potassium, magnesium, calcium, aluminum, etc., or salts with organic bases such as methylamine, ethylamine, ethanolamine, lysine, ornithine, etc. The present invention further embraces various hydrates and solvates to the compounds or salts thereof of the invention as well as polymorphisms thereof.

Hereinafter, representative processes for producing the compounds of the present invention are described. In these processes, functional groups present in the starting materials or intermediates may be suitably protected with protective groups, depending upon the kind of functional group. In view of the preparation techniques, it may be advantageous to protect the functional groups with groups that can readily be reverted to the original functional group. When required, the protective groups are removed to give the desired products. Examples of such functional groups are amino, hydroxy, carboxy groups, etc. Examples of the groups which may be used to protect these functional groups are shown in, e.g., Greene and Wuts, "Protective Groups in Organic Synthesis", second edition.

The general procedure for synthesizing pyrazolo[3,4-b]quinolin-5-one compounds is illustrated as follows:



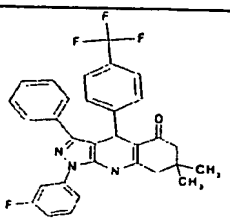
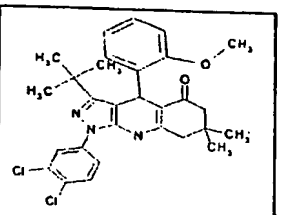
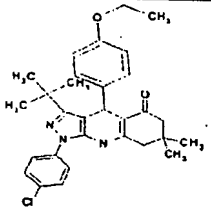
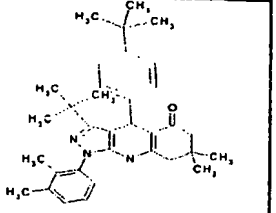
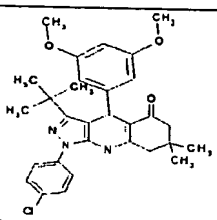
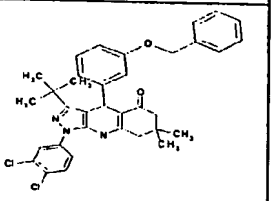
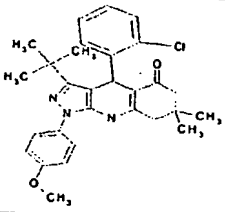
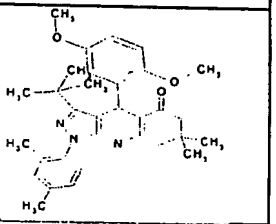
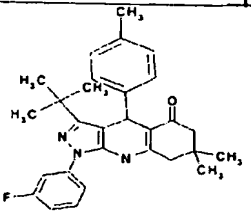
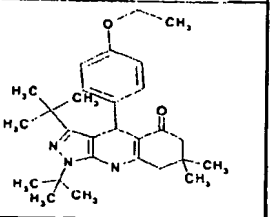
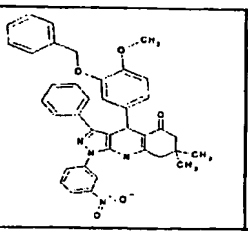
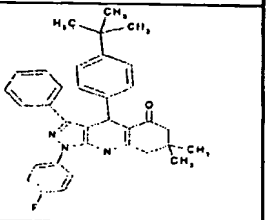
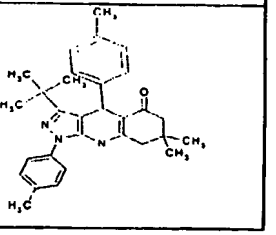
The reaction vessel was charged with aminopyrazole (1.0 mmol) dissolved in ethyl alcohol (10 mL). The appropriate aldehyde (1.0 mmol) and dimedone (1.0 mmol) were added to the above solution while stirring at room temperature. The reaction mixture was heated to 80 °C and refluxed for 6-8 h. The reaction vessel was then cooled to room temperature, and the solvent was removed under reduced pressure on a rotary evaporator. The residue was triturated with n-hexane in order to induce crystallization. The solid product was filtered off, washed abundantly with n-hexane and dried under ambient conditions. Yield: 30-75 %. Purity: 90-95 %.

The desired compound of the present invention may also be prepared by functional group transformation methods well known to those skilled in the art, which may depend on the kind of substituent. The order of the reactions, or the like, may be appropriately changed in accordance with the aimed compound and the type of reaction to be employed. The other compounds of the present invention and starting compounds can be easily produced from suitable materials in the same manner as in the above processes or by methods well known to those skilled in the art. Each of the reaction products obtained by the aforementioned production methods are isolated and purified as the free base or salt thereof. The salt can be produced by usual salt forming methods. The isolation and purification steps are carried out by employing conventional

chemical techniques such as extraction, concentration, evaporation, crystallization, filtration, recrystallization, various types of chromatography and the like.

Various forms of isomers can be isolated by conventional procedures making use of physicochemical differences among isomers. For instance, racemic compounds can be separated by means of conventional optical resolution methods (e.g., by forming diastereomer salts with a conventional optically active acid such as tartaric acid, etc. and then optically resolving the salts) to give optically pure isomers. A mixture of diastereomers can be separated by conventional means, e.g., fractional crystallization or chromatography. In addition, an optical isomer can also be synthesized from an appropriate optically active starting compound.

Table 1 list the structure of representative compounds of the present invention.

964661		964247	
964127		964336	
964144		964260	
964076		964232	
964352		963977	
964721		963924	
		964028	

One embodiment of the present invention relates to compounds that inhibit the activity of PI 3-K alpha. The invention further provides methods of inhibiting PI 3-K alpha.activity, including methods of modulating the activity of the PI 3-K alpha isozyme in cells, especially leukocytes, cardiomyocytes, osteoclasts, and cancer cells. Of particular benefit are methods of modulating PI 3-K alpha activity in the clinical setting in order to ameliorate disease or disorders mediated by PI 3-K alpha activity. Thus, treatment of diseases or disorders characterized by excessive or inappropriate PI 3-K alpha activity can be treated through use of modulators of PI 3-K alpha according to the present invention.

The compounds of the present invention may also show inhibitive activity against other PI 3-K isoforms, including PI 3-K beta, gamma, and delta. Therefore, the present invention also provides methods enabling the further characterization of the physiological role of each PI 3-K isozyme. Moreover, the invention provides pharmaceutical compositions comprising PI 3-K inhibitors and method of manufacturing and using such PI 3-Kinhibitor compounds (or a pharmaceutical composition comprising the compound).

The methods described herein benefit from the use of compounds that inhibit, and preferably specifically inhibit, the activity of a PI 3-K isoform in cells, including cells in vitro, in vivo, or ex vivo. Cells useful in the methods include those that express endogenous PI 3-K, wherein endogenous indicates that the cells express PI 3-K absent recombinant introduction into the cells of one or more polynucleotides encoding a PI 3-K isoform polypeptide or a biologically active fragment thereof. Methods also encompass use of cells that express exogenous PI 3-K isoforms wherein one or more polynucleotides encoding a PI 3-K isoforms or a biologically active fragment thereof, have been introduced into the cell using recombinant procedures.

Of particular advantage, the cells can be in vivo, i.e., in a living subject, e.g., an animal or human, wherein a PI 3-K inhibitor can be used therapeutically to inhibit PI 3-K activity in the subject. Alternatively, the cells can be isolated as discrete cells or in a tissue, for *ex vivo* or *in vitro* methods. *In vitro* methods also encompassed by the invention can comprise the step of contacting a PI 3-K enzyme, or a biologically active fragment thereof, with an inhibitor compound of the invention. The PI 3-K enzyme can include a purified and isolated enzyme, wherein the enzyme is isolated from a natural source (e.g., cells or tissues that normally express a PI 3-K polypeptide absent modification by recombinant technology) or isolated from cells modified by recombinant techniques to express exogenous enzyme.

The relative efficacies of compounds as inhibitors of an enzyme activity (or other biological activity) can be established by determining the concentrations at which each

compound inhibits the activity to a predefined extent and then comparing the results. Typically, the preferred determination is the concentration that inhibits 50% of the activity in a biochemical assay, i.e., the 50% inhibitory concentration or "IC_{50}." IC_{50} determinations can be accomplished using conventional techniques known in the art. In general, an IC_{50} can be determined by measuring the activity of a given enzyme in the presence of a range of concentrations of the inhibitor under study. The experimentally obtained values of enzyme activity then are plotted against the inhibitor concentrations used. The concentration of the inhibitor that shows 50% enzyme activity (as compared to the activity in the absence of any inhibitor) is taken as the IC_{50} value. Analogously, other inhibitory concentrations can be defined through appropriate determinations of activity. For example, in some settings it can be desirable to establish a 90% inhibitory concentration, i.e., IC_{90}, etc.

The compounds of the present invention exhibit kinase inhibitory activity, especially PI 3-K inhibitory activity and therefore, can be utilized to inhibit abnormal cell growth in which PI 3-K plays a role. Thus, the compounds are effective in the treatment of disorders with which abnormal cell growth actions of PI 3-K are associated, such as restenosis, atherosclerosis, bone disorders, arthritis, diabetic retinopathy, psoriasis, benign prostatic hypertrophy, atherosclerosis, inflammation, angiogenesis, immunological disorders, pancreatitis, kidney disease, cancer, etc. In particular, the compounds of the present invention possess excellent cancer cell growth inhibiting effects and are effective in treating cancers, preferably all types of solid cancers and malignant lymphomas, and especially, leukemia, skin cancer, bladder cancer, breast cancer, uterine cancer, ovarion cancer, prostate cancer, lung cancer, colon cancer, pancreatic cancer, renal cancer, gastric cancer, brain tumors, etc.

Accordingly, the invention provides methods of characterizing the potency of a test compound as an inhibitor of the PI 3-K polypeptide, said method comprising the steps of (a) measuring activity of a PI 3-K polypeptide in the presence of a test compound; (b) comparing the activity of the PI3 polypeptide in the presence of the test compound to the activity of the PI 3-K polypeptide in the presence of an equivalent amount of a reference compound (e.g., a PI 3-Kα. inhibitor compound of the invention as described herein), wherein lower activity of the PI 3-K polypeptide in the presence of the test compound than in the presence of the reference compound indicates that the test compound is a more potent inhibitor than the reference compound, and higher activity of the PI 3-K polypeptide in the presence of the test compound than in the presence of the reference compound indicates that the test compound is a less potent inhibitor than the reference compound.

The invention further provides methods of characterizing the potency of a test compound as an inhibitor of the PI 3-K polypeptide, comprising the steps of (a) determining an amount of a control compound (e.g., a PI 3-Kα inhibitor compound of the invention as described herein) that inhibits an activity of a PI 3-K polypeptide by a reference percentage of inhibition, thereby defining a reference inhibitory amount for the control compound; (b) determining an amount of a test compound that inhibits an activity of a PI 3-K polypeptide by a reference percentage of inhibition, thereby defining a reference inhibitory amount for the test compound; (c) comparing the reference inhibitory amount for the test compound to the reference inhibitory amount for the control compound, wherein a lower reference inhibitory amount for the test compound than for the control compound indicates that the test compound is a more potent inhibitor than the control compound, and a higher reference inhibitory amount for the test compound than for the control compound indicates that the test compound is a less potent inhibitor than the control compound.

In one aspect, the method uses a reference inhibitory amount which is the amount of the compound than inhibits the activity of the PI 3-Kα polypeptide by 50%, 60%, 70%, or 80%. In another aspect the method employs a reference inhibitory amount that is the amount of the compound that inhibits the activity of the PI 3-K α polypeptide by 90%, 95%, or 99%. These methods comprise determining the reference inhibitory amount of the compounds in an in vitro biochemical assay, in an in vitro cell-based assay, or in an in vivo assay.

The invention further provides methods of identifying a negative regulator of PI 3-K α activity, comprising the steps of (i) measuring activity of a PI3 α polypeptide in the presence and absence of a test compound, and (ii) identifying as a negative regulator a test compound that decreases PI 3-K α activity and that competes with a compound of the invention for binding to PI 3-Kα Furthermore, the invention provides methods for identifying compounds that inhibit PI 3-K α activity, comprising the steps of (i) contacting a PI 3-Kα polypeptide with a compound of the invention in the presence and absence of a test compound, and (ii) identifying a test compound as a negative regulator of PI 3-K α activity wherein the compound competes with a compound of the invention for binding to PI 3-K α. The invention therefore provides a method for screening for candidate negative regulators of PI 3-K α activity and/or to confirm the mode of action of candidates such negative regulators. Such methods can be employed against other PI 3-K isoforms in parallel to establish comparative activity of the test compound across the isoforms and/or relative to a compound of the invention.

In these methods, the PI 3-K polypeptide can be a fragment of the peptide that exhibits kinase activity or a fragment from the binding domain that provides a method to identify allosteric modulators of the peptide. The methods can be employed in cells expressing PI 3-K

peptide or its subunits, either endogenously or exogenously. Accordingly, the polypeptide employed in such methods can be free in solution, affixed to a solid support, modified to be displayed on a cell surface, or located intracellularly. The modulation of activity or the formation of binding complexes between the PI 3-K polypeptide and the agent being tested then can be measured.

Human PI 3-K polypeptides are amenable to biochemical or cell-based high throughput screening (HTS) assays according to methods known and practiced in the art, including melanophore assay systems to investigate receptor-ligand interactions, yeast-based assay systems, and mammalian cell expression systems. For a review, see Jayawickreme and Kost, Curr Opin Biotechnol, 8:629-34 (1997). Automated and miniaturized HTS assays also are comprehended as described, for example, in Houston and Banks, Curr Opin Biotechnol, 8:734-40 (1997). Such HTS assays are used to screen libraries of compounds to identify particular compounds that exhibit a desired property. Any library of compounds can be used, including chemical libraries, natural product libraries, and combinatorial libraries comprising random or designed oligopeptides, oligonucleotides, or other organic compounds.

The present invention also provides a method for inhibiting PI 3-K activity therapeutically or prophylactically. The method comprises administering an inhibitor of PI 3-K activity in an amount effective therefor in treating humans or animals who are or can be subject to any condition whose symptoms or pathology is mediated by PI 3-Kexpression or activity.

"Treating" as used herein refers to preventing a disorder from occurring in an animal that can be predisposed to the disorder, but has not yet been diagnosed as having it; inhibiting the disorder, i.e., arresting its development; relieving the disorder, i.e., causing its regression; or ameliorating the disorder, i.e., reducing the severity of symptoms associated with the disorder. "Disorder" is intended to encompass medical disorders, diseases, conditions, syndromes, and the like, without limitation.

The methods of the invention embrace various modes of treating an animal subject, preferably a mammal, more preferably a primate, and still more preferably a human. Among the mammalian animals that can be treated are, for example, companion animals (pets), including dogs and cats; farm animals, including cattle, horses, sheep, pigs, and goats; laboratory animals, including rats, mice, rabbits, guinea pigs, and nonhuman primates, and zoo specimens. Nonmammalian animals include, for example, birds, fish, reptiles, and amphibians.

In one aspect, the method of the invention can be employed to treat subjects therapeutically or prophylactically who have or can be subject to an inflammatory disorder. One aspect of the present invention derives from the involvement of PI 3-K in mediating aspects of

the inflammatory process. Without intending to be bound by any theory, it is theorized that, because inflammation involves processes are typically mediated by leukocyte (e.g., neutrophils, lymphocyte, etc.) activation and chemotactic transmigration, and because PI 3-K can mediate such phenomena, antagonists of PI 3-K can be used to suppress injury associated with inflammation.

"Inflammatory disorder" as used herein can refer to any disease, disorder, or syndrome in which an excessive or unregulated inflammatory response leads to excessive inflammatory symptoms, host tissue damage, or loss of tissue function. "Inflammatory disorder" also refers to a pathological state mediated by influx of leukocytes and/or neutrophil chemotaxis.

"Inflammation" as used herein refers to a localized, protective response elicited by injury or destruction of tissues, which serves to destroy, dilute, or wall off (sequester) both the injurious agent and the injured tissue. Inflammation is notably associated with influx of leukocytes and/or neutrophil chemotaxis. Inflammation can result from infection with pathogenic organisms and viruses and from noninfectious means such as trauma or reperfusion following myocardial infarction or stroke, immune response to foreign antigen, and autoimmune responses. Accordingly, inflammatory disorders amenable to the invention encompass disorders associated with reactions of the specific defense system as well as with reactions of the nonspecific defense system.

As used herein, the term "specific defense system" refers to the component of the immune system that reacts to the presence of specific antigens. Examples of inflammation resulting from a response of the specific defense system include the classical response to foreign antigens, autoimmune diseases, and delayed type hypersensitivity response mediated by T-cells. Chronic inflammatory diseases, the rejection of solid transplanted tissue and organs, e.g., kidney and bone marrow transplants, and graft versus host disease (GVHD), are further examples of inflammatory reactions of the specific defense system.

The term "nonspecific defense system" as used herein refers to inflammatory disorders that are mediated by leukocytes that are incapable of immunological memory (e.g., granulocytes, and macrophages). Examples of inflammation that result, at least in part, from a reaction of the nonspecific defense system include inflammation associated with conditions such as adult (acute) respiratory distress syndrome (ARDS) or multiple organ injury syndromes; reperfusion injury; acute glomerulonephritis; reactive arthritis; dermatoses with acute inflammatory components; acute purulent meningitis or other central nervous system inflammatory disorders such as stroke; thermal injury; inflammatory bowel disease; granulocyte transfusion associated syndromes; and cytokine-induced toxicity.

"Autoimmune disease" as used herein refers to any group of disorders in which tissue injury is associated with humoral or cell-mediated responses to the body's own constituents. "Allergic disease" as used herein refers to any symptoms, tissue damage, or loss of tissue function resulting from allergy. "Arthritic disease" as used herein refers to any disease that is characterized by inflammatory lesions of the joints attributable to a variety of etiologies. "Dermatitis" as used herein refers to any of a large family of diseases of the skin that are characterized by inflammation of the skin attributable to a variety of etiologies. "Transplant rejection" as used herein refers to any immune reaction directed against grafted tissue, such as organs or cells (e.g., bone marrow), characterized by a loss of function of the grafted and surrounding tissues, pain, swelling, leukocytosis, and thrombocytopenia.

The therapeutic methods of the present invention include methods for the treatment of disorders associated with inflammatory cell activation. "Inflammatory cell activation" refers to the induction by a stimulus (including, but not limited to, cytokines, antigens or auto-antibodies) of a proliferative cellular response, the production of soluble mediators (including but not limited to cytokines, oxygen radicals, enzymes, prostanoids, or vasoactive amines), or cell surface expression of new or increased numbers of mediators (including, but not limited to, major histocompatability antigens or cell adhesion molecules) in inflammatory cells (including but not limited to monocytes, macrophages, T lymphocytes, B lymphocytes, granulocytes (i.e., polymorphonuclear leukocytes such as neutrophils, basophils, and eosinophils), mast cells, dendritic cells, Langerhans cells, and endothelial cells). It will be appreciated by persons skilled in the art that the activation of one or a combination of these phenotypes in these cells can contribute to the initiation, perpetuation, or exacerbation of an inflammatory disorder.

The present invention enables methods of treating arthritic diseases, such as rheumatoid arthritis, monoarticular arthritis, osteoarthritis, gouty arthritis, spondylitis; Behcets disease; sepsis, septic shock, endotoxic shock, gram negative sepsis, gram positive sepsis, and toxic shock syndrome; multiple organ injury syndrome secondary to septicemia, trauma, or hemorrhage; ophthalmic disorders such as allergic conjunctivitis, venereal conjunctivitis, uveitis, and thyroid-associated ophthalmopathy; eosinophilic granuloma; pulmonary or respiratory disorders such as asthma, chronic bronchitis, allergic rhinitis, ARDS, chronic pulmonary inflammatory disease (e.g., chronic obstructive pulmonary disease), silicosis, pulmonary sarcoidosis, pleurisy, alveolitis, vasculitis, emphysema, pneumonia, bronchiectasis, and pulmonary oxygen toxicity; reperfusion injury of the myocardium, brain, or extremities; fibrosis such as cystic fibrosis; keloid formation or scar tissue formation; atherosclerosis; autoimmune diseases, such as systemic lupus erythematosus (SLE), autoimmune thyroiditis,

multiple sclerosis, some forms of diabetes, and Reynaud's syndrome; and transplant rejection disorders such as GVHD and allograft rejection; chronic glomerulonephritis; inflammatory bowel diseases such as chronic inflammatory bowel disease (CIBD), Crohn's disease, ulcerative colitis, and necrotizing enterocolitis; inflammatory dermatoses such as contact dermatitis, atopic dermatitis, psoriasis, or urticaria; fever and myalgias due to infection; central or peripheral nervous system inflammatory disorders such as meningitis, encephalitis, and brain or spinal cord injury due to minor trauma; Sjogren's syndrome; diseases involving leukocyte diapedesis; alcoholic hepatitis; bacterial pneumonia; antigen-antibody complex mediated diseases; hypovolemic shock; Type I diabetes mellitus; acute and delayed hypersensitivity; disease states due to leukocyte dyscrasia and metastasis; thermal injury; granulocyte transfusion-associated syndromes; and cytokine-induced toxicity.

The method can have utility in treating subjects who are or can be subject to reperfusion injury, i.e., injury resulting from situations in which a tissue or organ experiences a period of ischemia followed by reperfusion. The term "ischemia" refers to localized tissue anemia due to obstruction of the inflow of arterial blood. Transient ischemia followed by reperfusion characteristically results in neutrophil activation and transmigration through the endothelium of the blood vessels in the affected area. Accumulation of activated neutrophils in turn results in generation of reactive oxygen metabolites, which damage components of the involved tissue or organ. This phenomenon of "reperfusion injury" is commonly associated with conditions such as vascular stroke (including global and focal ischemia), hemorrhagic shock, myocardial ischemia or infarction, organ transplantation, and cerebral vasospasm. To illustrate, reperfusion injury occurs at the termination of cardiac bypass procedures or during cardiac arrest when the heart, once prevented from receiving blood, begins to reperfuse.

With respect to the nervous system, global ischemia occurs when blood flow to the entire brain ceases for a period. Global ischemia can result from cardiac arrest. Focal ischemia occurs when a portion of the brain is deprived of its normal blood supply. Focal ischemia can result from thromboembolytic occlusion of a cerebral vessel, traumatic head injury, edema, or brain tumor. Even if transient, both global and focal ischemia can cause widespread neuronal damage. Although nerve tissue damage occurs over hours or even days following the onset of ischemia, some permanent nerve tissue damage can develop in the initial minutes following the cessation of blood flow to the brain. Ischemia also can occur in the heart from myocardial infarction and other cardiovascular disorders in which the coronary arteries have been obstructed as a result of atherosclerosis, thrombus, or spasm. Accordingly, the invention is believed to be useful for

treating cardiac tissue damage, particularly damage resulting from cardiac ischemia or caused by reperfusion injury, in mammals.

In another aspect, inhibitors of PI 3-K activity, such as the compounds of the present invention, can be employed in methods of treating diseases of bone, especially diseases in which osteoclast function is abnormal or undesirable. Accordingly, the use of the compounds of the present invention can be of value in treating osteoporosis, Paget's disease, and related bone resorption disorders.

In a further aspect, the invention includes methods of using PI 3-K inhibitory compounds to inhibit the growth or proliferation of cancer cells of hematopoietic origin, preferably cancer cells of lymphoid origin, and more preferably cancer cells related to or derived from B lymphocytes or B lymphocyte progenitors. Cancers amenable to treatment using the methods of the present invention include, without limitation, lymphomas, e.g., malignant neoplasms of lymphoid and reticuloendothelial tissues, such as Burkitt's lymphoma, Hodgkins'lymphoma, non-Hodgkins lymphomas, lymphocytic lymphomas and the like; multiple myelomas; as well as leukemias such as lymphocytic leukemias, chronic myeloid (myelogenous) leukemias, and the like.

In another aspect, the invention includes a method for suppressing the function of basophils and/or mast cells, and thereby enabling treatment of diseases or disorders characterized by excessive or undesirable basophil and/or mast cell activity. According to the method, a compound of the invention can be used that inhibits the expression or activity of phosphatidylinositol 3-kinase in the basophils and/or mast cells. Preferably, the method employs a PI 3-K inhibitor in an amount sufficient to inhibit stimulated histamine release by the basophils and/or mast cells. Accordingly, the use of such compounds can be of value in treating diseases characterized by histamine release, i.e., allergic disorders, including disorders such as chronic obstructive pulmonary disease (COPD), asthma, ARDS, emphysema, and related disorders.

A compound of the present invention can be administered as the neat chemical, but it is typically preferable to administer the compound in the form of a pharmaceutical composition or formulation. Accordingly, the present invention also provides pharmaceutical compositions that comprise a chemical or biological compound ("agent") that is active as a modulator of PI 3-K activity and a biocompatible pharmaceutical carrier, adjuvant, or vehicle. The composition can include the agent as the only active moiety or in combination with other agents, such as oligo- or polynucleotides, oligo- or polypeptides, drugs, or hormones mixed with excipient(s) or other pharmaceutically acceptable carriers. Carriers and other ingredients can be deemed

pharmaceutically acceptable insofar as they are compatible with other ingredients of the formulation and not deleterious to the recipient thereof.

Techniques for formulation and administration of pharmaceutical compositions can be found in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co, Easton, Pa., 1990. The pharmaceutical compositions of the present invention can be manufactured using any conventional method, e.g., mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, melt-spinning, spray-drying, or lyophilizing processes. However, the optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. Such formulations can influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the administered agent. Depending on the condition being treated, these pharmaceutical compositions can be formulated and administered systemically or locally.

The pharmaceutical compositions are formulated to contain suitable pharmaceutically acceptable carriers, and can optionally comprise excipients and auxiliaries that facilitate processing of the active compounds into preparations that can be used pharmaceutically. The administration modality will generally determine the nature of the carrier. For example, formulations for parenteral administration can comprise aqueous solutions of the active compounds in water-soluble form. Carriers suitable for parenteral administration can be selected from among saline, buffered saline, dextrose, water, and other physiologically compatible solutions. Preferred carriers for parenteral administration are physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiologically buffered saline. For tissue or cellular administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For preparations comprising proteins, the formulation can include stabilizing materials, such as polyols (e.g., sucrose) and/or surfactants (e.g., nonionic surfactants), and the like.

Alternatively, formulations for parenteral use can comprise dispersions or suspensions of the active compounds prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, and synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions can contain substances that increase the viscosity of the suspension, such as sodium carboxymethylcellulose, sorbitol, or dextran. Optionally, the suspension also can contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Aqueous polymers that provide pH-sensitive solubilization and/or sustained release of the active agent also can be used as coatings or matrix structures, e.g., methacrylic polymers,

such as the EUDRAGIT.RTM. series available from Rohm America Inc. (Piscataway, N.J.). Emulsions, e.g., oil-in-water and water-in-oil dispersions, also can be used, optionally stabilized by an emulsifying agent or dispersant (surface active materials; surfactants). Suspensions can contain suspending agents such as ethoxylated isostearyl alcohols, polyoxyethlyene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, gum tragacanth, and mixtures thereof.

Liposomes containing the active agent also can be employed for parenteral administration. Liposomes generally are derived from phospholipids or other lipid substances. The compositions in liposome form also can contain other ingredients, such as stabilizers, preservatives, excipients, and the like. Preferred lipids include phospholipids and phosphatidyl cholines (lecithins), both natural and synthetic. Methods of forming liposomes are known in the art. See, e.g., Prescott (Ed.), Methods in Cell Biology, Vol. XIV, p. 33, Academic Press, New York (1976).

The pharmaceutical compositions comprising the agent in dosages suitable for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art. The preparations formulated for oral administration can be in the form of tablets, pills, capsules, cachets, dragees, lozenges, liquids, gels, syrups, slurries, elixirs, suspensions, or powders. To illustrate, pharmaceutical preparations for oral use can be obtained by combining the active compounds with a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Oral formulations can employ liquid carriers similar in type to those described for parenteral use, e.g., buffered aqueous solutions, suspensions, and the like.

Preferred oral formulations include tablets, dragees, and gelatin capsules. These preparations can contain one or more excipients, which include, without limitation:

a) diluents, such as sugars, including lactose, dextrose, sucrose, mannitol, or sorbitol;

b) binders, such as magnesium aluminum silicate, starch from corn, wheat, rice, potato, etc.;

c) cellulose materials, such as methylcellulose, hydroxypropylmethyl cellulose, and sodium carboxymethylcellulose, polyvinylpyrrolidone, gums, such as gum arabic and gum tragacanth, and proteins, such as gelatin and collagen;

d) disintegrating or solubilizing agents such as cross-linked polyvinyl pyrrolidone, starches, agar, alginic acid or a salt thereof, such as sodium alginate, or effervescent compositions;

e) lubricants, such as silica, talc, stearic acid or its magnesium or calcium salt, and polyethylene glycol;

f) flavorants and sweeteners;

g) colorants or pigments, e.g., to identify the product or to characterize the quantity (dosage) of active compound; and

h) other ingredients, such as preservatives, stabilizers, swelling agents, emulsifying agents, solution promoters, salts for regulating osmotic pressure, and buffers.

Gelatin capsules include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating such as glycerol or sorbitol. Push-fit capsules can contain the active ingredient(s) mixed with fillers, binders, lubricants, and/or stabilizers, etc. In soft capsules, the active compounds can be dissolved or suspended in suitable fluids, such as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

Dragee cores can be provided with suitable coatings such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures.

The pharmaceutical composition can be provided as a salt of the active agent. Salts tend to be more soluble in aqueous or other protonic solvents than the corresponding free acid or base forms. Pharmaceutically acceptable salts are well known in the art. Compounds that contain acidic moieties can form pharmaceutically acceptable salts with suitable cations. Suitable pharmaceutically acceptable cations include, for example, alkali metal (e.g., sodium or potassium) and alkaline earth (e.g., calcium or magnesium) cations.

Compounds of structural formula (I) that contain basic moieties can form pharmaceutically acceptable acid addition salts with suitable acids. For example, Berge et al. describe pharmaceutically acceptable salts in detail in J Pharm Sci, 66:1 (1977). The salts can be prepared in situ during the final isolation and purification of the compounds of the invention or separately by reacting a free base function with a suitable acid.

Representative acid addition salts include, but are not limited to, acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorolsulfonate, digluconate, glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate (isothionate), lactate, maleate, methanesulfonate or sulfate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, phosphate or hydrogen phosphate, glutamate, bicarbonate, p-toluenesulfonate, and undecanoate. Examples of acids that can be employed to form pharmaceutically acceptable acid addition salts include, without limitation, such inorganic acids as hydrochloric acid, hydrobromic acid, sulfuric acid, and phosphoric acid, and such organic

acids as oxalic acid, maleic acid, succinic acid, and citric acid.

In light of the foregoing, any reference to compounds of the present invention appearing herein is intended to include compounds of structural formula (I)-(III), [WHAT ARE THESE?] as well as pharmaceutically acceptable salts and solvates, as well as prodrugs, thereof.

Compositions comprising a compound of the present invention formulated in a pharmaceutically acceptable carrier can be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Accordingly, there also is contemplated an article of manufacture, such as a container comprising a dosage form of a compound of the invention and a label containing instructions for use of the compound. Kits are also contemplated under the invention. For example, the kit can comprise a dosage form of a pharmaceutical composition and a package insert containing instructions for use of the composition in treatment of a medical condition. In either case, conditions indicated on the label can include treatment of inflammatory disorders, cancer, etc.

Pharmaceutical compositions comprising an inhibitor of PI 3-K activity can be administered to the subject by any conventional method, including by parenteral and enteral techniques. Parenteral administration modalities include those in which the composition is administered by a route other than through the gastrointestinal tract, for example, intravenous, intraarterial, intraperitoneal, intramedullary, intramuscular, intraarticular, intrathecal, and intraventricular injections. Enteral administration modalities include, for example, oral (including buccal and sublingual) and rectal administration. Transepithelial administration modalities include, for example, transmucosal administration and transdermal administration. Transmucosal administration includes, for example, enteral administration as well as nasal, inhalation, and deep lung administration; vaginal administration; and rectal administration. Transdermal administration includes passive or active transdermal or transcutaneous modalities, including, for example, patches and iontophoresis devices, as well as topical application of pastes, salves, or ointments. Parenteral administration also can be accomplished using a high-pressure technique.

Surgical techniques include implantation of depot (reservoir) compositions, osmotic pumps, and the like. A preferred route of administration for treatment of inflammation can be local or topical delivery for localized disorders such as arthritis, or systemic delivery for distributed disorders, e.g., intravenous delivery for reperfusion injury or for systemic conditions such as septicemia. For other diseases, including those involving the respiratory tract, e.g., chronic obstructive pulmonary disease, asthma, and emphysema, administration can be

accomplished by inhalation or deep lung administration of sprays, aerosols, powders, and the like.

For the treatment of neoplastic diseases, especially leukemias and other distributed cancers, parenteral administration is typically preferred. Formulations of the compounds to optimize them for biodistribution following parenteral administration would be desirable. The PI 3-K inhibitor compounds can be administered before, during, or after administration of chemotherapy, radiotherapy, and/or surgery.

Moreover, the therapeutic index of the PI 3-K.delta. inhibitor compounds can be enhanced by modifying or derivatizing the compounds for targeted delivery to cancer cells expressing a marker that identifies the cells as such. For example, the compounds can be linked to an antibody that recognizes a marker that is selective or specific for cancer cells, so that the compounds are brought into the vicinity of the cells to exert their effects locally, as previously described (see for example, Pietersz et al., Immunol Rev, 129:57 (1992); Trail et al., Science, 261:212 (1993); and Rowlinson-Busza et al., Curr Opin Oncol, 4:1142 (1992)). Tumor-directed delivery of these compounds enhances the therapeutic benefit by minimizing potential nonspecific toxicities that can result from radiation treatment or chemotherapy. In another aspect, PI 3-K inhibitor compounds and radioisotopes or chemotherapeutic agents can be conjugated to the same anti-tumor antibody.

For the treatment of bone resorption disorders or osteoclast-mediated disorders, the PI 3-K inhibitors can be delivered by any suitable method. Focal administration may be desirable, such as by intraarticular injection. In some cases, it may be desirable to couple the compounds to a moiety that can target the compounds to bone. For example, a PI 3-K inhibitor can be coupled to compounds with high affinity for hydroxyapatite, which is a major constituent of bone. This can be accomplished, for example, by adapting a tetracycline-coupling method developed for targeted delivery of estrogen to bone (Orme et al., Bioorg Med Chem Lett, 4(11):1375-80 (1994)).

To be effective therapeutically in modulating central nervous system targets, the agents used in the methods of the invention should readily penetrate the blood brain barrier when administered peripherally. Compounds that cannot penetrate the blood brain barrier, however, can still be effectively administered by an intravenous route.

As noted above, the characteristics of the agent itself and the formulation of the agent can influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the administered agent. Such pharmacokinetic and pharmacodynamic information can be collected through preclinical *in vitro* and *in vivo* studies, later confirmed in humans during the

course of clinical trials. Thus, for any compound used in the method of the invention, a therapeutically effective dose can be estimated initially from biochemical and/or cell-based assays. Then, the dosage can be formulated in animal models to achieve a desirable circulating concentration range that modulates PI 3-Kexpression or activity. As human studies are conducted, further information will emerge regarding the appropriate dosage levels and duration of treatment for various diseases and conditions.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures using cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the "therapeutic index," which typically is expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices, i.e., the toxic dose is substantially higher than the effective dose, are preferred. The data obtained from such cell culture assays and additional animal studies can be used in formulating a range of dosages for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity.

For the methods of the present invention, any effective administration regimen regulating the timing and sequence of doses can be used. Doses of the agent preferably include pharmaceutical dosage units comprising an effective amount of the agent. As used herein, "effective amount" refers to an amount sufficient to modulate PI 3-K expression or activity and/or derive a measurable change in a physiological parameter of the subject through administration of one or more of the pharmaceutical dosage units.

Exemplary dosage levels for a human subject are of the order of from about 0.001 milligram of active agent per kilogram body weight (mg/kg) to about 100 mg/kg. Typically, dosage units of the active agent comprise from about 0.01 mg to about 10,000 mg, preferably from about 0.1 mg to about 1,000 mg, dcpending upon the indication, route of administration, etc. Dcpending on the route of administration, a suitable dose can be calculated according to body weight, body surface area, or organ size. The final dosage regimen will be determined by the attending physician in view of good medical practice, considering various factors that modify the action of drugs, e.g., the agent's specific activity, the identity and severity of the disease state, the responsiveness of the patient, the age, condition, body weight, sex, and diet of the patient, and the severity of any infection.

Additional factors that can be taken into account include time and frequency of administration, drug combinations, reaction sensitivities, and tolerance/response to therapy.

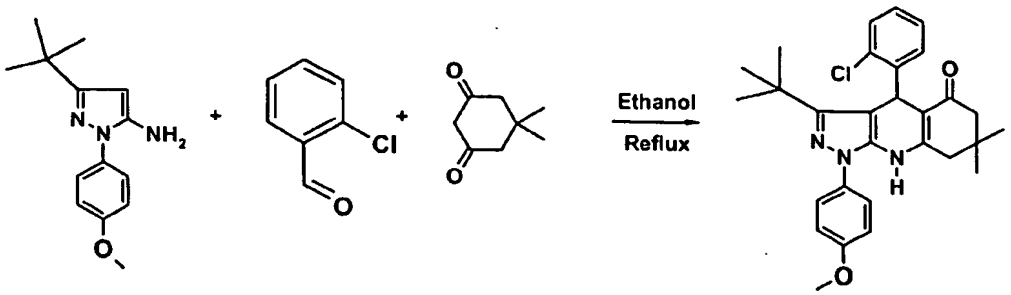
Further refinement of the dosage appropriate for treatment involving any of the formulations mentioned herein is done routinely by the skilled practitioner without undue experimentation, especially in light of the dosage information and assays disclosed, as well as the pharmacokinetic data observed in human clinical trials. Appropriate dosages can be ascertained through use of established assays for determining concentration of the agent in a body fluid or other sample together with dose response data.

The frequency of dosing will depend on the pharmacokinetic parameters of the agent and the route of administration. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Accordingly, the pharmaceutical compositions can be administered in a single dose, multiple discrete doses, by continuous infusion, as sustained release depots, or combinations thereof, as required to maintain the desired minimum level of the agent. Short-acting pharmaceutical compositions (i.e., short half-life) can be administered once a day or more than once a day (e.g., two, three, or four times a day). Long acting pharmaceutical compositions might be administered every 3 to 4 days, every week, or once every two weeks. Pumps, such as subcutaneous, intraperitoneal, or subdural pumps, can be preferred for continuous infusion.

The following Examples are provided to further aid in understanding the invention, and pre-suppose an understanding of conventional methods well-known to those persons having ordinary skill in the art to which the examples pertain. Such methods are described in detail in numerous publications including, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), Ausubel et al. (Eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994); and Ausubel et al. (Eds.), Short Protocols in Molecular Biology, 4th ed., John Wiley & Sons, Inc. (1999). The particular materials and conditions described hereunder are intended to exemplify particular aspects of the invention and should not be construed to limit the reasonable scope thereof.

Example 1. Synthesis and Characterization of 0964076

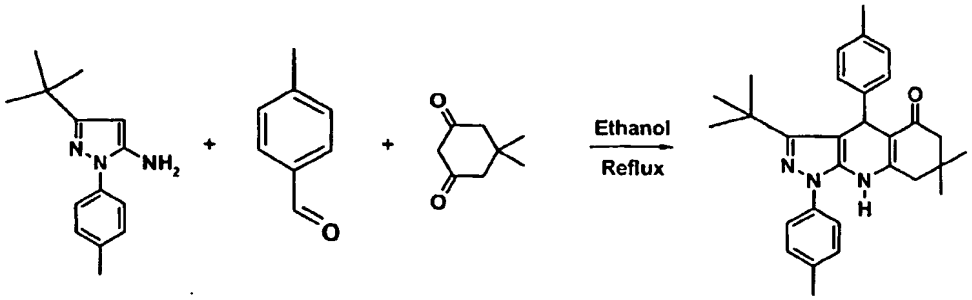
3-*t*-butyl-4-(2-chlorophenyl)-7,7-dimethyl-1-(4-methoxyphenyl)-4,7,8,9-tetrahydro-1*H*-pyrazolo[3,4-b]quinolin-5(6*H*)-one



The reaction vessel was charged with 1-(4-methoxyphenyl)-3-t-butyl-5-aminopyrazole (500 mg, 2.03 mmol) was dissolved in ethyl alcohol (20 mL). Then, (2-chloro-benzaldehyde (218 mL, 2.43 mmol) and dimedone (285 mg, 1.0 mmol) was added to the above solution with stirring at room temperature. The reaction mixture was heated to 80 °C and refluxed for 6 h. Then the reaction vessel was cooled to room temperature, and the solvent was removed under reduced pressure on a rotary evaporator. The residue was triturated with n-hexane in order to induce the crystallization. The solid product was redissolved and further purified by column chromatography yielding a pure product (110 mg).

1H NMR (CDCl3): 0.8, 1.02, 1.1, 1.23, 2.03, 2.14, 3.85, 5.67, 6.32, 7.02, 7.14, 7.23, 7.44.

Example 2. Synthesis and Characterization of 0964028
3-*t*-butyl-4-(4-methylphenyl)-7,7-dimethyl-1-(4-methylphenyl)-4,7,8,9-tetrahydro-1*H*-pyrazolo[3,4-b]quinolin-5(6*H*)-one



The react+ion vessel was charged with 1-(4-methylphenyl)-3-t-butyl-5-aminopyrazole (180 mg, 0.78 mmol) was dissolved in ethyl alcohol (10 mL). Then, *p*-tolualdehyde (110 mL, 0.94 mmol) and dimedone (110 mg, 0.78 mmol) was added to the above solution with stirring at room temperature. The reaction mixture was heated to 80 °C and refluxed for 6 h. Then the reaction vessel was cooled to room temperature, and the solvent was removed under reduced pressure on a rotary evaporator. The residue was triturated with n-hexane in order to induce the

crystallization. The solid product (178 mg) was filtered off, washed and dried under ambient conditions.

1H NMR (CDCl3): 0.82, 1.03, 1.14, 1.23, 2.25, 2.41, 5.40, 6.22, 7.00, 7.18, 7.31, 7.43.

Example. 3 Isolation of Recombinant PI 3-K

Recombinant heterodimeric PI 3-Kα, consisting of a p110 catalytic subunit and a GST-tagged p85 regulatory subunit, was expressed in Sf9 cells using a baculovirus expression system. Expression constructs were obtained from the lab of Dr. Alex Toker, Harvard University. The method is well known to those skilled in the art and is also described in Stoyanov et al., Science 269, 690–693 (1995).and Stoyanova et al., Biochem. J. 324 :489–495. (1997).

The harvested cell pellet was re-suspended in 3 ml of Buffer A (20mM Tris pH 7.0, 150mM NaCl, 10mM EDTA, 20mM Sodium Fluoride, 5mM Sodium Pyrophosphate, 10% Glycerol, 0.1% Igapal) containing protease inhibitors (1mM PMSF, 1mM $NaVO_3$, Leupeptin 1ug/ml, Pepstatin 1ug/ml.) The suspension was incubated for 1hour at 4°C with rotation to break the cells, and then vortexed gently to ensure cell lysis. The solution was centrifuged at 14,000g for 15 minutes, and the supernatant was diluted by the addtion of 10ml of Buffer A. The diluted supernatant was added to 3ml of Glutathione-agarose resin (Pharmacia) pre-equilibrated in Buffer A, and incubated for 1 hour at 4°C with rotation. The resin was poured into a column and washed with 35ml of Buffer A, and the protein was eluted using 10mM Glutathione in Buffer A. Twenty, 0.5ml fractions were collected and the presence of protein was assessed on 12% SDS-PAGE Tris Glycine gel (Invitrogen). Fractions containing target protein were pooled and concentrated using a Microsep 30K concentrator (Pall-Gelman). The concentrated protein was diluted with 3 ml of Final Buffer (20mM Tris pH 7.4, 100mM NaCl, 1mM EDTA) and concentrated twice more to remove any detergent. The protein was diluted in 50% glycerol and stored at -20°C.

Example 4. PI 3-K Activity Assay and Screen for PI 3-K Inhibitors

Vectors for expression of GST-GRP1-PH were obtained from Mark Lemmon, University of Pennsylvania. (Kavran, et al., J Biol Chem, 273:30497-30508 (1998)). Protein expression and purification from *E. coli* was carried out as follows: A LB/amp plate was streaked from a frozen glycerol stock of *E coli* containing the expression vector and grown overnight at 37°C. A single colony was picked and inoculated into 20 ml of LB media containing 100ug/ml ampicillin, and grown overnight. The overnight culture was added to 1 Liter of LB media

containing 100ug/ml ampicillin and grown until the O.D. 600 was between 0.8-1.0. Protein expression was induced by the addition of 0.1 mM IPTG, and cultures continued to grow overnight at 37°C. Cells were harvested by centrifugation at 4,000g for 20 minutes. Pellets were stored frozen at -80°C until protein purification was carried out. The purification of GST-tagged protein was performed as follows: the pellets were resupended in 25 ml of Buffer A (50mM Tris pH 7.5, 1mM BME, 1mM EDTA, 1mM EGTA, 1mM NaVO3, 50mM Sodium Fluoride, 5mM Sodium Pyrophosphate, 0.27M Sucrose) with protease inhibitors (1mM PMSF, 0.5ug/ml Leupeptin, 0.7ug/ml Pepstatin). The cells were lysed by sonication for 3 minutes, and Triton x-100 was added to a final concentration of 0.01%. The mixture was clarified by centrifugation at 10,000rpm for 15 minutes. The supernatant was mixed with 5 ml Glutathione-agarose resin (Amersham), pre-equilibrated in Buffer A. The protein was allowed to bind to the resin for 1 hour at 4°C with rotation. The resin was transferred into a column and washed with 30 ml of Buffer A. The protein was eluted using 10mM Glutathione (Sigma) in Buffer A. Twenty, 1ml fractions were collected and protein levels assessed by SDS-PAGE on 12% Tris-Glycine gels (Invitrogen). The fractions containing purified protein were pooled and stored at – 20 C°.

PI 3-kinase reactions were performed in a reaction buffer containing 5 mM HEPES, pH 7, 2.5 mM $MgCl_2$, and 25 μM ATP, containing 50 ng of recombinant PI 3-K with 10 picomoles of diC_8 $PI(4,5)P_2$ (Echelon Biosciences) as the substrate. The reactions were allowed to proceed at room temperature for 1-3 hours, then quenched by the addition of EDTA to a final concentration of 10mM. The final reaction volumes were 10 μl. The compounds to be tested for inhibition were added to a final concentration of 1 μM from stocks in DMSO. The final concentration of DMSO was 1%.

Conversion of the substrate to $PI(3,4,5)P_3$ was determined using a competition assay using Amplified Luminescent Proximity Homogeneous Assay (ALPHA®) technology developed by Perkin Elmer. 0.25 picomoles of recombinant GST-Grp1-PH domain protein and 0.25 picomoles of biotinylated diC_6 $PI(3,4,5)P_3$ (Echelon Biosciences) were added to each reaction mixture. Donor and Acceptor beads from the AlphaScreen® GST (Glutathione-S-Transferase) Detection Kit (PerkinElmer) were added to a final concentration of 20 μg/ml. The final volume was 25 μl. The reactions were incubated at 37 °C for two hours, and the luminescent signal was read on a Fusion α microplate reader. Percent inhibition of enzyme activity was determined by comparison to no enzyme (100 % inhibition) and DMSO alone (0% inhibition) controls.

Example 5. Determination of IC_{50} for PI 3-K Inhibitors

A library of potential PI 3-K inhibitors was tested for activity against PI 3-K α in the following manner. From the active compounds identified, twelve were selected as representatives from different chemical groups present in the library and subjected to further analysis. IC_{50} values were determined for the sixteen selected compounds. Enzyme activity assays were performed as previously described, in the presence of a range of compound concentrations to allow determination of IC_{50} values. Enzyme activity and percent inhibition was determined using the AlphaScreen® luminescent assay as previously described. Table 2 shows % inhibition at 1 μM in an in vitro PI 3-K assay against recombinant PI 3-K α for selected compounds as well as the IC_{50} values. These inhibitors may also show activity against other PI 3-K isoforms, including PI 3-K beta, gamma, and delta.

Table 2. In Vitro inhibition of PI 3-K activity at 1 μM and IC50 values (nM) for selected compounds.

Compound Number	%Inhibition @ 1 μM in vitro	IC_{50} (nM)
0142183	53	636.3
0900664	100	84.25
0900661	100	409.2
0964721	100	87.55
0963985	88	371.2
0964144	99	358.9
0963924	79	398.5
0916462	69.6	446.1
2012339	99	44.44
2011011	94	163.7
2004359	76	929.2
0914276	83	331.5

Example 6 .Characterization of Effects of PI 3-K Inhibitors on Cancer Cells

Selected compounds were tested for selective activity against paired ovarian cancer cell lines. The SKOV3 cell line is not altered in PI 3-K signaling and should be less sensitive to the anti-proliferative effects produced by treatment with PI 3-K inhibitors, while the OVCAR3 cell line, which is altered in PI 3-K signaling, should be sensitive. SKOV3 cells were seeded in 96-well cell culture plates (Greiner) at a density of 20,000 cells per well in McCoys 5A media (GibcoBRL) with 10% fetal calf serum and 20 mM L-glutamine. OVCAR3 cells were seeded at

a density of 15,000 cells per well in RPMI 1640 media (GibcoBRL) containing 20 mM l-glutamine, 0.01 mg/ml bovine insulin, 10 mM Hepes pH 7.4, 1 mM sodium pyruvate, 2.5 g/L glucose, and 20 % fetal calf serum. The compounds were added to cell media to a final concentration of 1 μM, and the cells were grown in the presence of the compounds for 48 hours. Viability was determined using a MTT cell proliferation assay (R and D Systems) and comparison to DMSO alone controls (100% viability). Compounds which result in reduced viability may act either by inhibiting cell proliferation or by inducing apoptosis (programmed cell death). Compounds representative of the 096 and 093 structural groups within the library showed selective effects on cell proliferation and viability. These results are shown in Table 3 and Figure 1.

Table 3. Effects of selected compounds on paired ovarian cancer cell lines.

	% Viability at 1 mM	
Compound	SKOV3	OVCAR3
0142183	100	94
0900664	100	96
0900661	100	99
0964721	100	97
0963985	72	36
0964144	94	84
0963924	100	85
0916462	88	75
2012339	97	83
2011011	91	83
2004359	88	90
0914276	100	97

Compounds present in the library which had been identified as PI 3-K inhibitors using the *in vitro* screen, and which were also structurally related to the compounds of the present invention that showed cell-specific effects on viability, were tested for activity against the paired cell lines. Many of these also show similar selective effects on cell growth. Table 4 summarizes the results of two separate cell proliferation experiments for selected compounds of the present invention.

Table 4. Summary of two different experiments in which selected of the present invention were tested for selective effects on paired ovarian cancer cell lines.

Compound	Trial 1		Trial 2		Average	average
	SKOV-3	OVCAR3	SKOV-3	OVCAR3	SKOV3	OVCAR-3
0964661	99	36	77.1	58.9	88.05	47.45
0964076	100	41	92.9	53.2	96.45	47.1
0964127	100	42	100	76.2	100	59.1
0964144	100	54	100	66.2	100	60.1
0964352	93	33	74	52.9	83.5	42.95
0964028	100	42	99.1	45.4	99.55	43.7
0964247	100	42	100	43.7	100	42.85
0964336	96	32	83	56	89.5	44
0964260	93	41	85	53	89	47
0964232	98	45	100	71	99	58
0963977	98	41	81.7	59.6	89.85	50.3
0963924	100	61	100	66.4	100	63.7

PI 3-K inhibitors which show this activity profile may be effective against a number of tumor cell lines and tumor types in which PI 3-K signaling is altered, either by amplification of PI 3-K activity, or by mutations which effect regulation of PI 3-K activity, including mutations in the tumor suppressor PTEN. These include breast, prostate, colon, and ovarian cancers.

It is to be understood that the above-referenced arrangements are only illustrative of the application for the principles of the present invention. Numerous modifications and alternative arrangements can be devised without departing from the spirit and scope of the present invention. While the present invention has been shown in the drawings and fully described above with particularity and detail in connection with what is presently deemed to be the most practical and preferred embodiment(s) of the invention, it will be apparent to those of ordinary skill in the art that numerous modifications can be made without departing from the principles and concepts of the invention as set forth herein.

ABSTRACT OF THE DISCLOSURE

Compounds inhibiting phosphatidylinositol 3-kinase activity, and methods of preparing and using thereof in treating diseases, such as disorders of immunity and inflammation, in which PI 3-K plays a role in leukocyte function are disclosed. Compounds inhibiting PI 3-K activity and methods of using PI 3-K inhibitory compounds to inhibit cancer cell growth or proliferation are also provided.

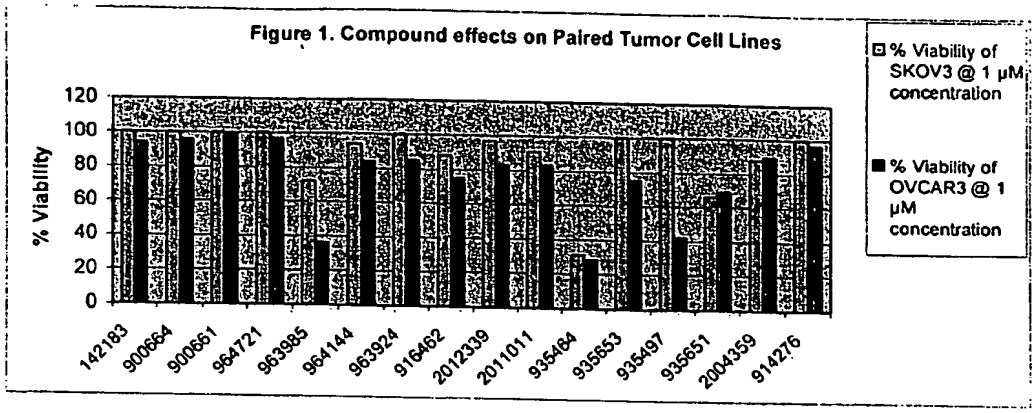


Figure 1. Compound effects on Paired Tumor Cell Lines
% Viability
120
100
80
60
40
20
0
142183
900664
900651
964721
963985
964144
963924
916462
2012339
2011011
935484
935553
935497
935651
2004359
914276
% Viability of SKOV3 @ 1 µM concentration
% Viability of OVCAR3 @ 1 µM concentration

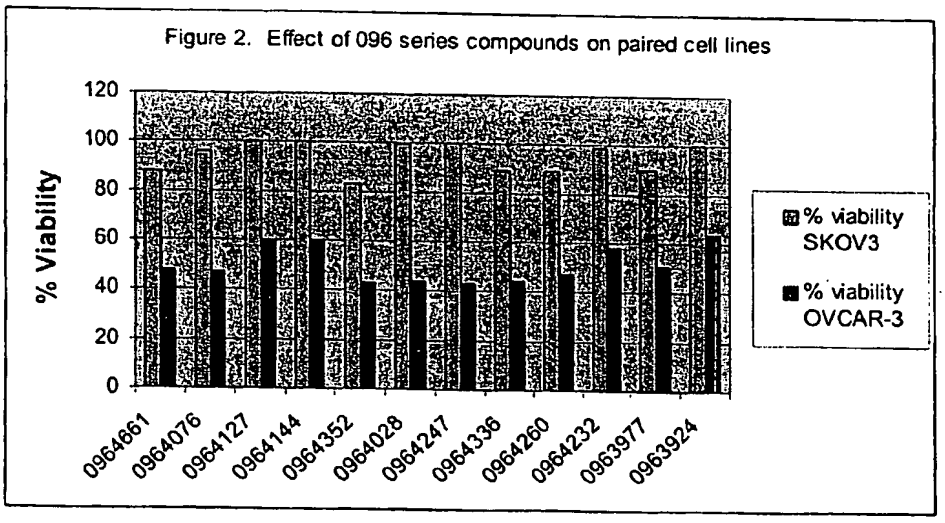


Figure 2. Effect of 096 series compounds on paired cell lines
% Viability
120
100
80
60
40
20
0
0964661
0964076
0964127
0964144
0964352
0964028
0964247
0964336
0964260
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0963977
0963924
% viability SKOV3
% viability OVCAR-3

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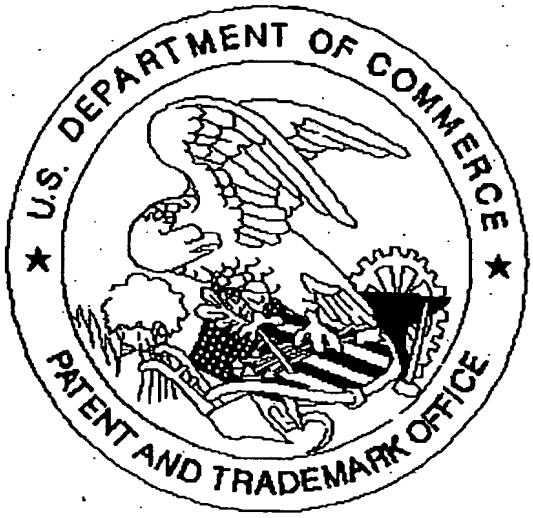

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